INFRARED NEURAL STIMULATION OF THALAMOCORTICAL BRAIN SLICES *IN VITRO*

By

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CHAPTER I

INTRODUCTION

Overview of Nervous System

The nervous system is divided into two separate systems, the central nervous system (CNS) and the peripheral nervous system (PNS) as seen in Figure 1 (A. C. Guyton and J. E. Hall, 2006). In general, the CNS functions by receiving signals from the PNS, processing these signals, and then generates an appropriate response. The PNS transmits signals to the CNS by transducing stimuli into electro-chemical signals. Both the PNS and the CNS contain neurons, the functional unit of the nervous system, as well as support cells to ensure proper functioning of the neurons.

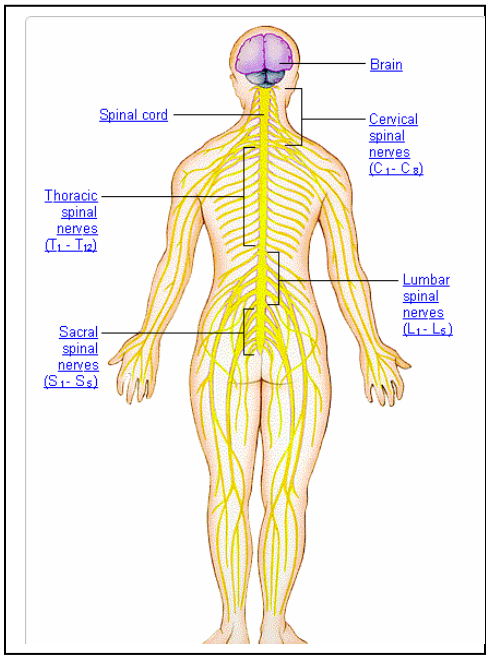

Figure 1: Central and Peripheral Nervous system (A. C. Guyton and J. E. Hall, 2006).

The CNS is composed of two main sections, the spinal cord and the brain. The spinal cord is located in the vertebrae column of the skeleton, and functions by performing lower level processing, such as invoking a movement in a reflex arc, and transmitting signals to the appropriate higher processing areas of the brain. The brain is located in the cranial cavity of the head and is connected to the spinal cord through the magnum foramen. The spinal cord and cranial nerves relay signals to different regions of the brain from sensory nerves located in the PNS. The brain processes these signals and determines the appropriate response to the stimulus or originates signals to induce movement or other autonomic action without a stimulus (A. C. Guyton and J. E. Hall, 2006).

The PNS contains all neurons that lie outside the CNS. Sensory or afferent neurons relay signals transduced from external stimuli to the CNS for processing. Motor or efferent neurons relay signals from the CNS to the appropriate target organs to illicit a response. Motor and sensory neurons are coupled together to form nerves which originate directly from the spinal cord and brain. Each nerve serves as a central location to transmit information to and from specific regions of the body. Additionally, the distal ends of some sensory neurons participate in the transduction of external stimuli into the electro-chemical signals used by neurons to carry signals to the CNS (A. C. Guyton and J. E. Hall, 2006).

The neuron is the functional unit if the nervous system. A basic neuron contains two processes, a dendrite and an axon, which are connected to a cell body. Neurons are classified into three different categories, multipolar, bipolar, and unipolar (Figure 2). A

multipolar neuron has multiple dendrites that branch out from the cell body and synapse with many different axons.

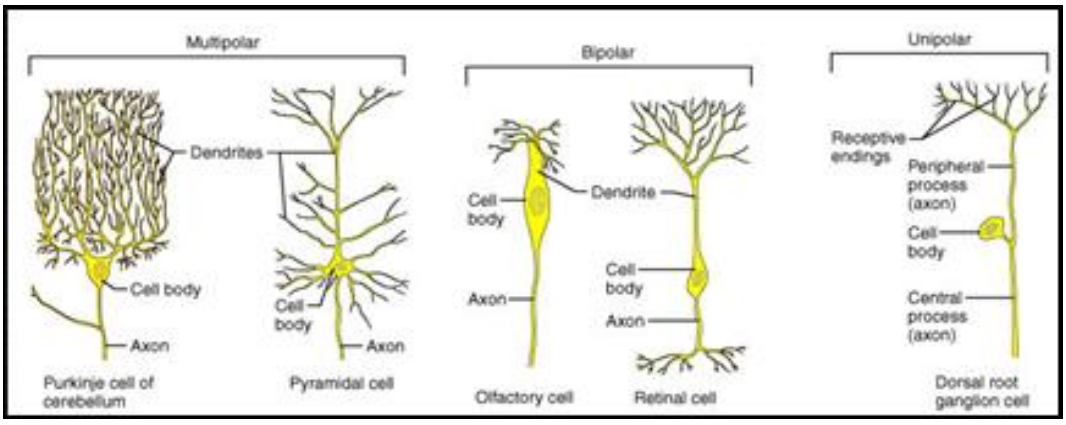

Figure 2: Representation of each type of neuron with example cell type (A. C. Guyton and J. E. Hall, 2006).

A bipolar neuron has a single dendrite connected to the cell body on one pole, while the neuron's axon is connected to the cell body on the opposite pole. A unipolar neuron connects the dendrite directly to the axon with the cell body being located at the connection of the two processes. Signals are relayed to a neuron by depolarizing the membrane through a synapse on the dendrite or cell body of the neuron (R. Plonsey and R. Barr, 2000; B. Hille, 2001). Neurotransmitter released at the synapse binds to a receptor protein on the postsynaptic membrane of the target neuron which causes an ion channel to open. When the ion channel opens, a negative or positive charged ion rushes into the cell either depolarizing or hyperpolarizing the membrane. Depolarization at a synapse evokes a transient electro-chemical signal which propagates down the neuronal dendrite and cell body towards the axon hillock by opening voltage gated ion channels through temporal depolarization of adjacent sections of membrane (K. Cole, 1968; R.

Plonsey and R. Barr, 2000). If the transient signal reaches the axon hillock without losing a significant amount of energy, then an action potential will result and propagate down an axon to the next synapse. On the other hand, hyperpolarization of the membrane at a synapse causes inhibition of the creation of an action potential. Inhibition of action potentials only occurs in the regions of the brain where information is processed; however depolarization and action potential generation is seen in both the PNS and CNS. Some axons in the PNS and CNS have sections covered in myelin sheaths to serve as insulation to help action potentials propagate down the length of the axon efficiently (R. Plonsey and R. Barr, 2000). Myelin is produced by specialized cells called oligodendrocytes, myelin production cells in the CNS, and Schwann cells, myelin production cells in the PNS. Each forms myelin in small sections which give rise to the nodes of Ranvier or small regions of exposed cellular membrane. The nodes of Ranvier allow for saltatory conduction down the axon where action potentials are rapidly forced to the next node by the insulation of the myelin sheaths. The saltatory conduction allows for the fast propagation speeds needed for complex movements and fast reaction times (R. Plonsey and R. Barr, 2000). Other supporting cells in the CNS include astrocytes and microglia. Astrocytes serve as scaffolding and helps facilitate delivery of nutrients to ensure proper functioning of neurons in the CNS. Migrolia cells help to defend neurons from microorganisms and removes dead neuronal cells. The PNS also contains satellite cells which perform the same function as astrocytes by supporting neurons located in the PNS (R. Plonsey and R. Barr, 2000; A. C. Guyton and J. E. Hall, 2006).

Electrical Stimulation and Detection

Traditionally, neuronal activity has been studied using electrical stimulation and detection methods. To stimulate a neuron, an electrode is placed on or near the neuronal tissue. A small current is applied to the electrode to depolarize the membrane of the neuron evoking an action potential, which propagates down the axon towards additional synapses or to target tissue. A typical electrical stimulation of a sciatic nerve is seen in Figure 3 (J. Wells et al., 2005a). Electrical stimulation can be used to stimulate other tissues such as skeletal muscles, cardiac muscle, and smooth muscle in addition to direct neural stimulation.

Electrical stimulation can be applied in the brain in the cortex and evoked responses can be observed downstream in specific regions of the body. Also, electrical stimulation has been used to pace the heart to eliminate abnormal rhythms and electrical stimulation has been used to inhibit abnormal signals in the brain to eliminate tremors commonly seen in Parkinson's disease and essential tremor.

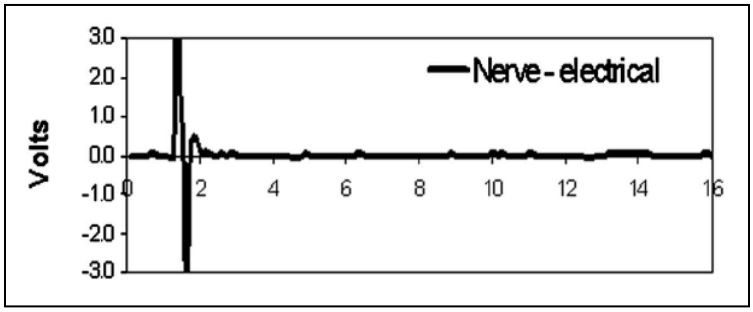

Figure 3: Electrical recording of single electrical stimulation. Notice large stimulation artifact due to incident electrical energy and small electrical depolarization following artifact (J. Wells et al., 2005a).

Electrical stimulation has also been used to map areas of function in the brain during surgery and has been used to identify hyperactive areas in the nervous system to diagnose different disorders (R. Plonsey and R. Barr, 2000). Although electrical stimulation has been established as the gold standard for stimulating neurons in basic and clinical applications, electrical stimulation has numerous limitations. The electrical field applied from an electrode propagates outward from the electrode in a spherical motion while decaying exponentially with distance. This phenomenon results in poor spatial resolution due to the recruitment of multiple neural networks or neurons when stimulation is applied. Also, the electrode must come into contact with at least the extracellular fluid or the tissue itself. To obtain single cellular electrical stimulation, the electrode must impale the cell which results in apoptosis once the electrode is removed. Additionally, each pulse of electrical energy applied to the neural tissue causes an artifact that can mask neural response to the stimulation, and can induce responses that are not physiologically accurate (E. Civillico and D. Contreras, 2005; J. Wells et al., 2005a; J. Wells et al., 2005b; C. Huang et al., 2006).

Electrical detection is the most common modality used to detect intrinsic activity as well as activity evoked using electrical stimulation. Similarly to electrical stimulation, an electrode must be placed on or near the tissue to detect changes in the membrane potential (R. Plonsey and R. Barr, 2000). However, the electrode is limited to the immediate area and cannot detect electrical activity in distal regions from the electrode. Also, traditional electrical detection methods are limited by low throughput and have large amounts of background noise that can mask neural responses. Intracellular recording or single cell recordings can only be made by impaling the neuron which will

cause apoptosis when the electrode is removed (C. Peterson et al., 2002; E. Civillico and D. Contreras, 2005; C. Huang et al., 2006). Due to the inherent limitations of traditional electricophysiological techniques there is a need to develop new modalities which addresses these limitations. Recently infrared neural stimulation methods have been developed which offer an alternative to electrical methods.

Infrared Neural Stimulation

Recently, neural stimulation has been performed using infrared light to evoke action potentials that are artifact free (J. Wells et al., 2005b; A. D. Izzo et al., 2006; I. U. Teudt et al., 2007). The mechanism behind optical stimulation involves establishing a radiant heat gradient through heating the cellular membrane of the neuron from absorption of infrared light. The radiant heat gradient evokes an artifact free action potential that is similar in shape to action potentials created electrically as can be seen in Figure 4 (J. Wells et al., 2005a; J. Wells et al., 2007b; J. Wells et al., 2007a). INS stimulation results in a smaller magnitude action potential as compared to electrical stimulation. The smaller magnitude observed during optical stimulation is due to lower number of axons being stimulated in the sciatic nerve. The lower number of recruited axons demonstrates the spatial precision of optical stimulation allowing researchers to stimulate individual muscle groups whereas electrical stimulation recruits all axons resulting in stimulation of all muscles downstream from the stimulation sight (J. Wells et al., 2005a; J. Wells et al., 2007b; J. Wells et al., 2007a).

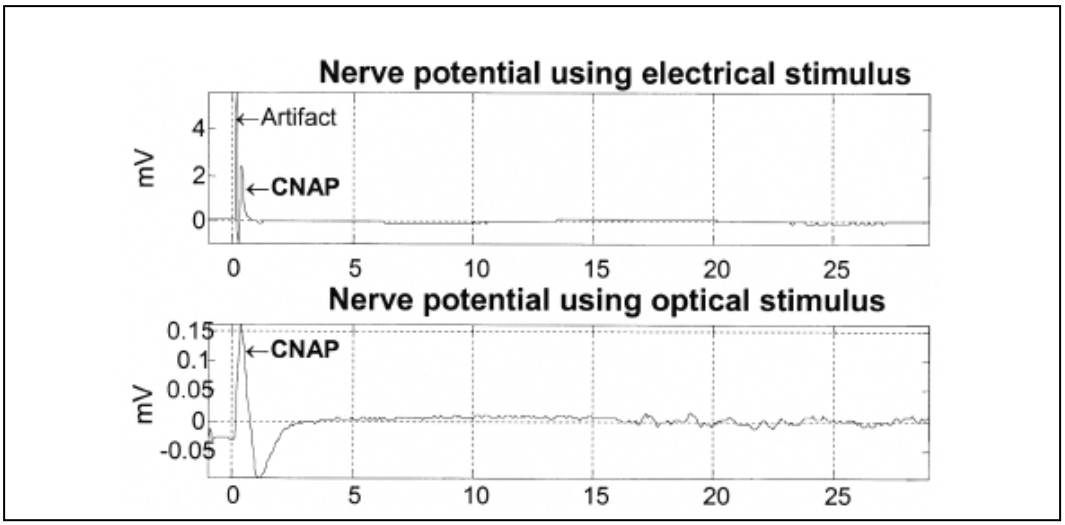

Figure 4: Electrical and INS stimulation from same experiment. CNAP = Compound Neural Action Potential (J. Wells et al., 2005a).

INS stimulation has been demonstrated in the rat sciatic nerve model using the following wavelengths that lie on peaks and valleys in the water absorption curve: 1.85, 2.1, 3.0, 4.0, 4.5, 5.0, and 6.1 μm at an optimal frequency of 2 Hz. The nerve to be stimulated is placed under the light with a radiant exposure ranging from 0.3 J/cm^2 to above 1.0 J/cm^2 to establish stimulation threshold (J. Wells et al., 2005a). Of these wavelengths, 1.85, 2.1 and 4.0 μm proved to have the highest safety margin based on analysis from histological studies focusing on determining the extent of thermal damage (J. Wells et al., 2005a; J. Wells et al., 2005b; J. Wells et al., 2007c). In addition to being spatially precise, the method also does not require a probe to touch tissue eliminating any risk of damaging neural tissue from physical contact (J. Wells et al., 2005a).

Current Applications of INS

Infrared neural stimulation has quickly been applied to a number of different clinical applications where a need for high spatial precision is needed to effectively diagnose or treat a medical condition. Recent developments include INS of the cochlea and auditory nerve (A. D. Izzo et al., 2006), the facial nerve (I. U. Teudt et al., 2007), and the cavernous nerve surrounding the prostate (M. F. Nathaniel et al., 2008). Stimulation of the cochlea and auditory nerve is aimed at improving hearing for deaf individuals while stimulation of the facial and cavernous nerves is focused on improving diagnostic abilities of the clinician during tumor resection.

Studies involving INS of the cochlea and the auditory nerve have shown high spatial precision with low radiant energy thresholds (A. D. Izzo et al., 2006; A. D. Izzo et al., 2007). Further studies showed a pulse width of 35 μs and a repetition rate of 13 Hz could be used to efficiently stimulate the gerbil auditory system for up to 12 hours; however they were limited by the maximum repetition rate of the laser (A. D. Izzo et al., 2007). Recently, the same group showed the infrared evoked action potentials were detectable in the inferior colliculus thus verifying the optically induced signals reached the higher processing centers of the brain (L. Phillip et al., 2008). INS of the auditory system was shown to produce more spatially precise auditory maps when compared to electrical stimulation, and could possibly be used to improve the performance of hearing aids for patients with hearing impairment (A. D. Izzo et al., 2007; A. D. Izzo et al., 2008; L. Phillip et al., 2008).

Two peripheral nerve INS applications have recently been developed to be used to discriminate neural tissue from cancerous tissue margins during tumor resections. Safe

facial nerve stimulation has been shown to be feasible using 2.12 μm light at a repetition rate of 2 Hz. Similarly to the sciatic nerve studies, individual muscles in the face can be selectively stimulated. Successful development of this technique can help surgeons discriminate between tumorous tissue and neural tissue preventing facial paralysis (I. U. Teudt et al., 2007). Another diagnostic application is the INS of cavernous nerves surrounding the prostate. The cavernous nerve contains sympathetic and parasympathetic neurons which innervate the corpus cavernosum and control the erection of the penis. Researchers have shown an increase in blood pressure in the corpus cavernosum can be measured in response to INS of the cavernous nerve (M. F. Nathaniel et al., 2008). Cavernous nerve INS was the first application where a physical response could be measured from stimulating a sensory nerve, and the spatial precision of INS was demonstrated which proved feasibility for discriminating neural tissue from diseased prostate tissue during resection using INS.

A number of other groups have begun investigating uses of INS for different applications where high spatially precise and contact free stimulation are desired. Researchers hope to expand the auditory system stimulation to include vestibular stimulation, a technique that has been difficult to implement due to the poor spatial precision of electrical stimulation. Another application is the stimulation of the vagus nerve optically to prevent shock from developing after an acute inflammatory insult has been delivered to the body, a technique that has been performed using electrical stimulation. The aim of this study is to use INS to only stimulate the individual neural fibers of the vagus nerve which control the auto-immune response of the body. The method would prevent other complications from developing due to entire vagus nerve

stimulation seen with electrical stimulation. Interest has also developed for using INS to stimulate damaged peripheral nerves to restore function to paralyzed limbs in a machine interface system. The studies mentioned here are only a few possible applications of INS in the peripheral nervous system, and have all been developed over the past 3 years; however INS has not been optimized for central nervous system.

Motivation

Central nervous system stimulation is the natural next step in developing INS for use in the clinic. The properties of INS will improve on current electrical stimulation techniques used to treat and diagnose patients by providing a contact free stimulation modality with high spatial precision. Furthermore, clinicians and researchers will both benefit from no stimulation artifact being present on electrical recording traces revealing more biologically relevant. Implementation of INS in the central nervous system will allow clinicians to be more precise in their procedures to treat patients. Clinicians will be able to better discriminate critical cortical areas during tumor or epileptic cortical resections. Deep brain stimulation will have better spatial precision than electrical deep brain stimulating eliminating the debilitating effects of current spread from the procedure for the treatment of movement disorders (Parkinson's and Essential Tremor), pain management, and seizures. Furthermore, basic scientific research will benefit by allowing investigators to study biological potentials which were masked by the electrical stimulation artifact to better understand the signal processing of the nervous system. Significant potential exists for INS in the central nervous system for both clinical and

basic research which indicates the need to develop the optimal parameters for stimulating the brain.

Thalamocortical Brain Slices:

The complex organization of the brain has created difficulty in localizing signals using electrical techniques while performing electrical stimulation or INS. Researchers developed a method to dissect the brain into sections while preserving specific neuronal networks connecting the cortex to the deep nuclei of the brain allowing for direct study of action potential propagation in these networks (M. G. Blanton et al., 1989; A. Agmon and B. W. Connors, 1991; C. Q. Kao and D. A. Coulter, 1997). Furthermore, techniques have been developed to record intracellularly in the brain slice which has allowed researchers to study individual biological currents related to ion channels and neurotransmitters (M. G. Blanton et al., 1989; C. Q. Kao and D. A. Coulter, 1997). Agmon et al. has developed a model which isolates the somatosensory barrel cortex of the brain in the mouse and rat models while preserving the efferent and afferent connections to the thalamus maintaining a neuronal circuit which can be studied (A. Agmon and B. W. Connors, 1991). Additionally, the slicing process preserves a section of the hippocampus which can be used for obtaining intracellular recordings (C. Q. Kao and D. A. Coulter, 1997). This model is easily reproducible allowing multiple parametric investigations to be carried out in a short amount of time, and the network of neurons are visible under white light illumination while in the perfusion chamber (A. Agmon and B. W. Connors, 1991). These factors are beneficial for optimizing INS for the brain *in Vitro* which will be used to guide future studies *in Vivo*.

Hypothesis and Objectives:

Infrared neural stimulation has been well characterized in the rate sciatic nerve (J. Wells et al., 2007a) and the auditory system (A. D. Izzo et al., 2007); however no INS has been reported in the central nervous system. Previous attempts by our group, affiliated with the Vanderbilt Biomedical Engineering and Neurosurgery Departments, to stimulate cortex *in Vivo* have failed to illicit a measurable response. The reason for these failed attempts is thought to be due to a combination of the high spatial precision of INS and the inability of electrical recording techniques to detect the INS evoked signal; therefore the hypothesis of this research assumes infrared light can efficiently stimulate neural tissue in the brain using an *in Vitro* thalamocortical brain slice model. The brain slice model allows for the preservation of a well characterized three neuron network (A. Agmon and B. W. Connors, 1991). Since the pathways of these networks are known, the electrical recording electrodes can be placed in regions downstream from the INS site to improve the probability of detecting the optically induced action potentials. We suspect that INS will be able to illicit a one to one response as was seen in the studies of the rat sciatic nerve and will result in artifact free electrical recordings (J. Wells et al., 2007b).

The goal of this study can be summarized into three distinct objectives. Feasibility of performing INS in the central nervous system will be shown using the *in Vitro* thalamocortical brain slice model. Once feasibility has been shown, then a parametric study will be conducted to determine the optimal wavelength, repetition rate, and spot size for implementing INS in the brain slice model. The final objective of the brain slice study will be to establish feasibility in implementing INS with electrical

intracellular recording techniques. Next the research will move into an *in Vivo* model to execute a more detailed parametric study to optimize INS for safe efficient implementation in the brain. Additional feasibility studies will investigate the use of optical imaging techniques for detection of brain activation for the *in Vivo* studies. Once the optimal parameters for *in Vivo* INS have been established, then efficacy of INS will be demonstrated for chronic stimulation. Finally, the clinical efficacy will be investigated for both diagnostic and therapeutic application as a clinical tool.

This thesis focuses on proving feasibility of INS in the thalamocortical brain slice, characterization of the optimal parameters to perform INS in the thalamocortical brain slice, and showing feasibility to perform INS while using intracellular recording methods. The results from this thesis will guide the future studies designed to progress the development of INS towards use as a clinical tool for treatment and diagnostic applications.

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CHAPTER II

INFRARED NEURAL STIMULATION OF THALAMOCORTICAL BRAIN SLICES IN VITRO

Introduction

Neural stimulation is the process of activating neurons using an external source to evoke action potential propagation down an axon. Electrical, chemical, thermal, optical, and mechanical methods have all been reported to stimulate neurons in both the central nervous system (CNS) and the peripheral nervous system (PNS) (S. D. Schoonhoven R, 1991). Electrical stimulation is currently the gold standard for the activating neurons, and functions by increasing the transmembrane potential to activate voltage-gated ion channels which induce action potential propagation down the axon of a neuron (K. Cole, 1968; R. Plonsey and R. Barr, 2000; B. Hille, 2001; C. Huang et al., 2006). However, electrical stimulation lacks spatial precision due to the inherent electrical field propagation which results in the recruiting of multiple unwanted neuronal fibers. Additionally, electrical stimulation induces a recording artifact which can mask neuronal signals resulting from the simulation (M. KC et al., 1982; E. Civillico and D. Contreras, 2005).

Infrared neural stimulation (INS) was recently presented as a novel alternate means for the stimulation of peripheral nerves. Studies show that infrared light, with wavelengths ranging from 2.1 to 4.0 μm, can be used to selectively excite muscles innervated by the sciatic nerve *in vivo*. Resultant recordings show no stimulation artifact and histological studies confirm no damage was caused by the irradiation of the nerve

with infrared light. Thus INS offers a method for artifact-free, damage-free, contact-free and spatially selective method of neural activation (J. Wells et al., 2005a; J. Wells et al., 2005b). These advantages make optical stimulation a viable alternative to standard electrical stimulation.

However, to date, INS research has been confined to the peripheral nerve, imploring the question of whether it is possible to stimulate the brain using this technique. INS has the potential to improve on current electrical techniques used in research and medicine by providing high spatial precision stimulation and electrical artifact free recordings in a contact free method. These properties of INS will allow for better understanding of how the brain processes information in basic studies and will provide neurosurgeons the ability to provide higher quality care to patients undergoing cortical resections or deep brain stimulation procedures. Thus the next step in the development of infrared neural stimulation is to achieve and optimize stimulation in the CNS, specifically the brain.

The varying geometry and physiology of the brain implies that a different set of parameters will be needed to achieve stimulation in the CNS. Unlike the PNS where axons of neurons travel parallel in nerves, the brain is organized into a complex neuronal network. The complex organization of the brain has caused difficulty in detecting neuronal activation evoked by INS in an *in Vivo* preparation; thus the thalamocortical brain slice model was chosen for an *in Vitro* study to simply detection of INS evoked signals. The thalamocortical brain slice model preserves a three neuron network between cortical and thalamic neurons that reproduce action potential activity similar to that seen with *in vivo* animal studies (A. Agmon and B. W. Connors, 1991; C. Q. Kao and D. A.

Coulter, 1997). The three neuronal network of the thalamocortical brain slice allows for some organization and repeatability between individual preparations when optimizing infrared neural stimulation techniques. The purpose of this study was to prove feasibility and optimize the parameters of optical stimulation *in vitro* in the thalamocortical brain slice model.

Methods

Spraque-Dawley rats (21 to 35 days) were anesthetized with inhalation of 4% isoflurane for 1-1.5 min and were immediately decapitated. The brain was rapidly dissected from the cranial cavity, and the cerebellum was removed. A parasagittal cut was made at a 55 degree angle from the midline of the brain at 10 degree elevation to allow for correct orientation during slicing (A. Agmon and B. W. Connors, 1991). Thalamocortical slices were then cut to be between 400-500 μm thick using a Vibratome. During slice preparation, the brain tissue was bathed in an artificial cerebral spinal fluid (ACSF) containing the following concentrations (in mM): 124 NaCl , 5 KCl , 1.25 NaH_2PO_4, 2 $CaCl_2$, 1-2 $MgCl_2$ 26 $NaHCO_3$and 10 glucose. The ACSF was oxygenated using a 95% O_2 / 5% CO_2 gas concentration which maintain the tissue throughout slice preparation and experimentation (C. Q. Kao and D. A. Coulter, 1997). The thalamocortical slices were then placed in a similar oxygenated ACSF solution that was magnesium deficient to induce hyperactivity and the slices were allowed to incubate for one hour. After incubation, the slice was placed in a perfusion chamber at room temperature and perfused by oxygenated potassium deficient ACSF solution at a rate of 2.5 ml/min. Electrical recordings were made with glass patch electrodes (3-6 MΩ) pulled

from borosilicate glass capillaries (World Precision Instruments, Sarasota, FL) using a vertical Narishige PP-83 glass pipette puller, (Scientific Instrument Lab., Tokyo, Japan). The glass electrode was filled with a fluid containing the following constituent concentrations (in mM): cesium-potassium gluconate 110, $MgCl_2$ 2, Na-EGTA 1, $CaCl_2$ 0.1, MgATP 2, NaGTP 0.2 and HEPES 10. The glass electrode was placed onto the tissue using a micromanipulator to record extracellular potentials. Recording of all electrical signals were made with an Axopatch-200B amplifier (Axon Instruments, Foster City, CA) and the signal was digitized and analyzed using the Axon Digidata 1322A data acquisition system and pCLAMP8 software (Axon Instruments Inc).

All experiments were performed using the Vanderbilt University W.M. Keck Free Electron Laser Center (FEL). The FEL is a tunable laser source with a wavelength range between 2.5 μm and 10 μm. The spot size was measured using the razor edge technique to establish accurate radiant exposure. Initial feasibility studies used a wavelength of 4.00 μm due to its relatively low absorption. The initial frequencies (repetition rates) used were 15 and 30 Hz, and the spot size was maintained around 220 μm. The pulse width for all experiments was 5 μs and was not adjustable.

Wavelength optimization was performed using the following wavelengths: 2.51 μm, 3.65 μm, 4.00 μm, and 5.3 μm. Each wavelength was picked for its unique penetration depth ranging from approximately 200 μm –600 μm. INS at each wavelength was performed using 30 Hz and a spot size between 300 – 400 μm. Each wavelength was used to stimulate at least 3 separate viable slices at 4-6 locations on the (n=12-16). Laser stimulation was applied to the cortical area of the brain slice and the glass electrode was placed at least 2 mm away in the cortex. Threshold was determined when a visual

change in the baseline signal of the recording from the glass pipette electrode that could be eliminated by the application of the sodium channel blocker tetratodotoxin (TTX). A recording was made of the signal with a trigger trace generated by the FEL corresponding to pulse generation. Frequency (repetition rate) dependence was studied by performing a set of experiments with 3.65 μm light and varying frequency (30 Hz, 15 Hz, 10 Hz, 7.5 Hz, and 6 Hz) using 3 slices for each repetition rate with 4-6 stimulation sites dependent on slice quality (n=16-17). Consistent orientation was maintained by irradiating tissue 2 mm away from the recording electrode using the different frequencies identified above. Again TTX was used to establish biological origin of the recorded response. Spot size was studied using 3.65 μm light at 30 Hz with a spot size ranging from 140 μm to 510 μm, and INS was performed using the same protocol outlined for the frequency dependence study.

Intracellular recordings were attempted at 4 μm and 3.65 μm in the ventrobasal thalamus and cortical sections of the brain slice by applying positive pressure to the pipette as the probe was advanced slowly in the tissue towards the neurons. Once a whole cell patch was made, the tissue was irradiated with infrared light and the signal was recorded (M. G. Blanton et al., 1989).

A final set of experiments were performed to minimize stimulation thresholds by ablating approximately the first 50 μm of tissue. Experiments were carried out at 3.65 μm and 4.00 μm light at 30 Hz. Stimulation was carried out in the cortical area of the slice roughly 2 mm away from the recording electrode. Laser irradiance was increased to ablation threshold, and 6-8 pulses were applied to the tissue to remove the dead tissue.

Stimulation was then applied as described for the initial experiments with application of TTX to determine biological basis for signal generation.

Results

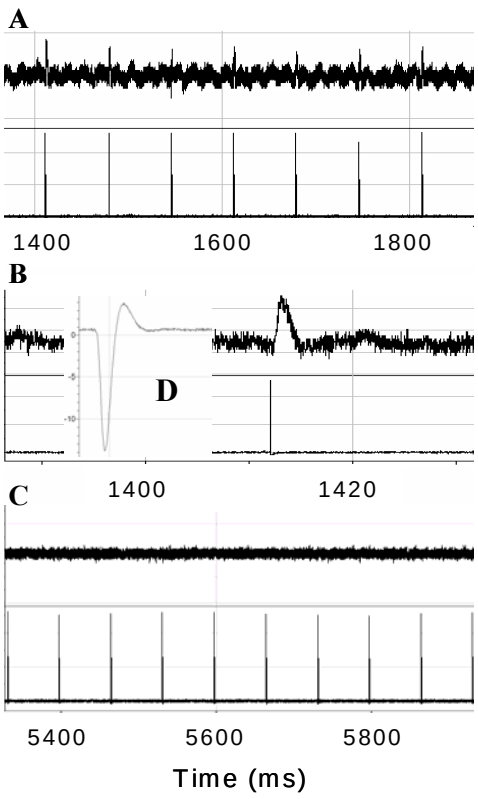

Figure 1: Infrared stimulation evokes neural spike activity in the thalamocortical slice as seen in extracellular recordings**.** A-C. Top trace shows recorded extracellular potentials; bottom trace represents timing of each pulse. A. Infrared light evoked action potentials. B. Zoomed trace on one spike. C. Lack of infrared evoked spike activity after TTX application. Laser parameters: 19.25 J/cm^2, 15 Hz, 220 μm spot size, 4.0 μm light. D. Single pulse of electrical stimulation using 10 mA current.

Initial feasibility of infrared neural stimulation was established using 4.0 μm at 15 Hz with a spot size of 220 μm. Figure 1 represents stimulation of a brain slice using 4.0

μm. The signal induced by the infrared light was frequency locked with the laser repetition rate. A zoomed view of the INS stimulation reveals no stimulation artifact that is commonly seen with electrical stimulation (Figure 1b). The signal induced by infrared light was eliminated once TTX was applied to the tissue (Figure 1c). Figure 1d represents a single action potential from electrical stimulation. Note the large stimulation artifact which is present during electrical stimulation (Figure 1d), but not present during INS (Figure 1b). Attempts to stimulate the thalamus, hippocampus, and other deep structures contained in the slice produced similar extracellular recordings as seen in Figure 1

Based on these results, wavelength dependence of INS was determined. INS was performed using wavelengths of light at 2.51 μm, 3.65 μm, 4.00 μm, and 5.3 μm. Each wavelength was found to successfully stimulate a brain slice and the signal was verified by the application of TTX. The optimal wavelength for INS with minimal radiant exposure was identified to be 3.65 μm (Figure 2). The average radiant exposure at 3.65 μm was 1.86 J/cm^2 with a standard deviation of +/- .4 J/cm^2, and the ablation threshold was determined to be approximately 1.1 J/cm^2.

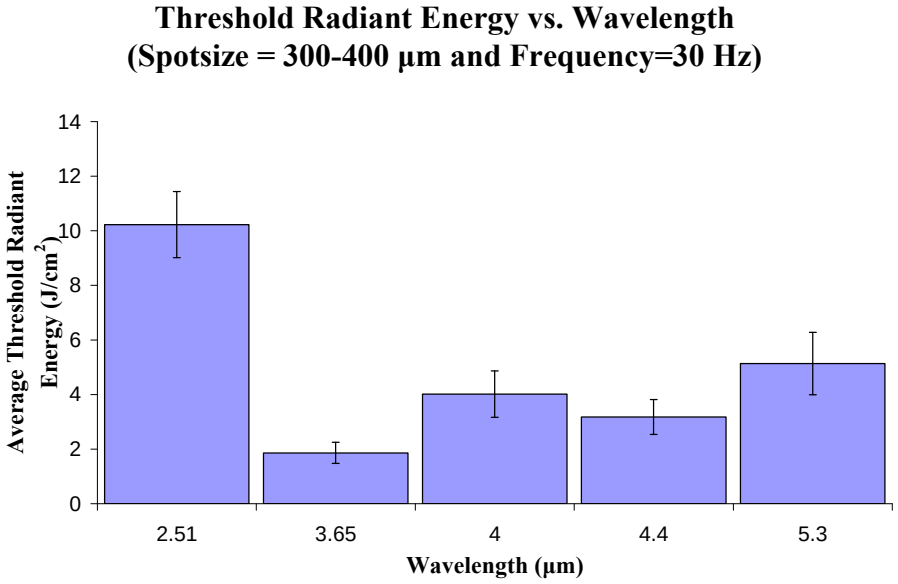

Figure 2: Infrared radiant energy levels at neural activation thresholds for different wavelengths**.** Laser parameters: Spot size = 300-400 µm, Freq.=30 Hz

Frequency dependence and spot size dependence was established using the optimal wavelength at 3.65 µm. The spot size was fixed between 300 – 340 µm for the frequency study. A frequency of 30 hertz produced the lowest radiant energies (~1.86 J/cm^2) needed for stimulation. Figure 3 demonstrates that threshold radiant energies increased as the repetition rate decreased, and the best frequency with the lowest radiant energy needed for INS was 30 HZ. It should be noted that this is the highest frequency possible with this laser.

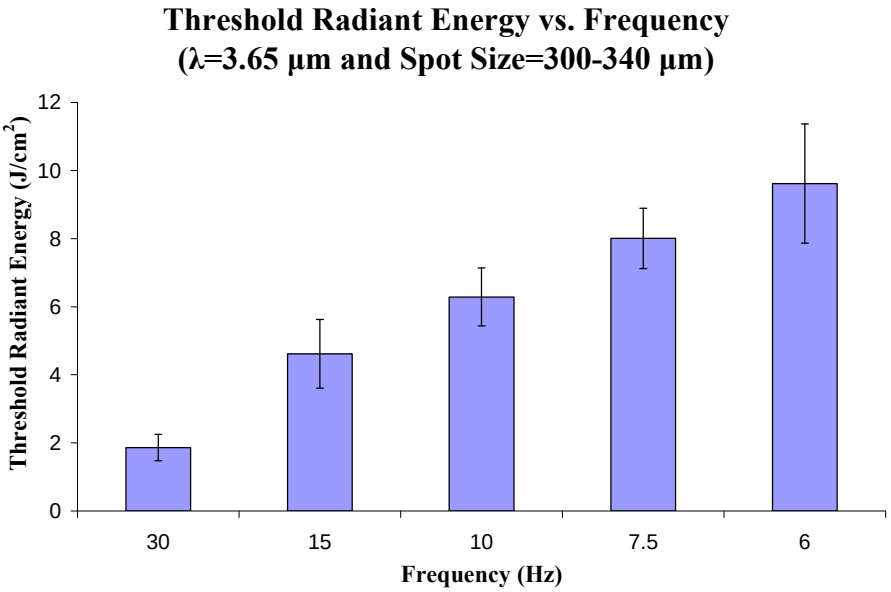

Figure 3: Increasing pulse frequency reduces radiant energy levels at neuronal activation thresholds**.** Laser parameters: λ=3.65 µm and Spot Size = 300-340 µm.

For the next part of the study, the repetition rate was fixed at 30 Hz and the spot size was varied to determine the optimal spot size. Results show a larger spot size reduces the threshold radiant energy needed to evoke extracellular potentials (Figure 4). The resultant curve exhibits a power relationship (R^2=.8966) . For all experiments at 3.65 µm where stimulation was observed, ablation occurred around 1.15 J/cm^2; however removal of the superficial dead layer of tissue (~50 µm) allowed for the threshold radiant energy to be dropped to 0.66 J/cm^2. The energy did not ablate or visibly damage the tissue when applied to regions not previously exposed to infrared energy, and TTX efficiently eliminated the infrared induced signal when applied to the tissue in the perfusion chamber.

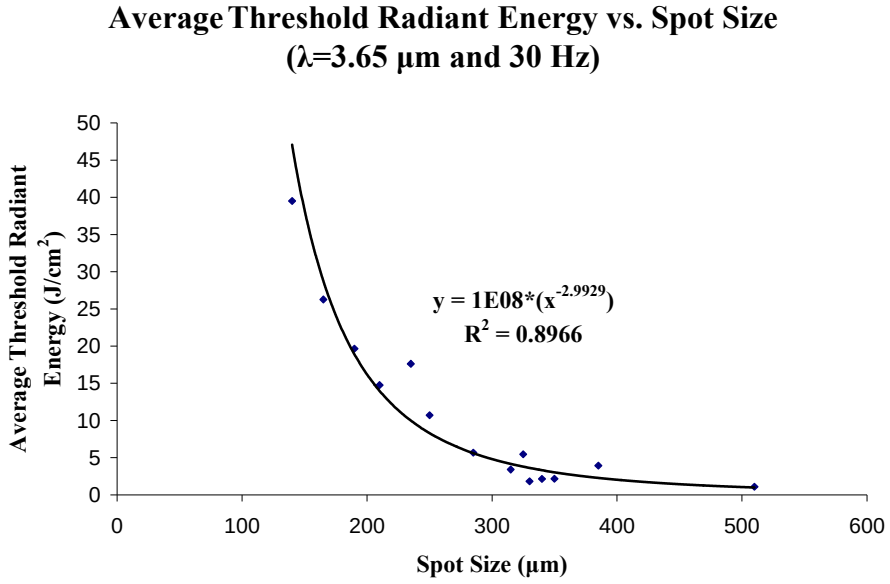

Figure 4: Increasing spot size reduces radiant energy levels at neuronal activation thresholds and exhibits a 3rd order power fit. Laser parameters: λ=3.65 µm .

Figure 5 represents an intracellular recording using 3.65 µm at 30 Hz. The trace shows an initial fast component followed by additional peaks in the intracellular potential trace. The additional peaks represent the infrared induced signal traveling through the neuronal networks providing feedback to the neuron where the recording was made. TTX was used to eliminate the signal to ensure no mechanical or thermal artifact existed in the signal.

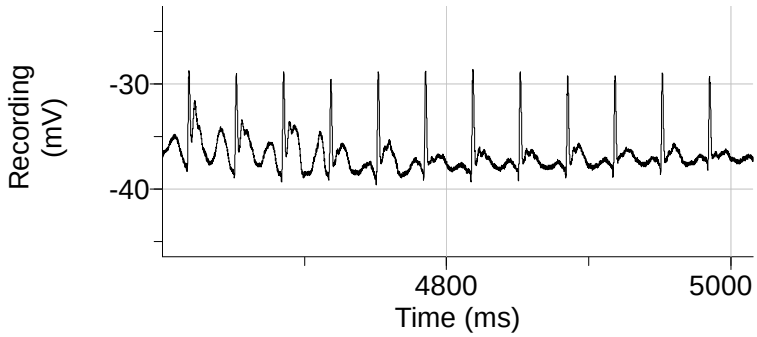

Figure 5: Infrared stimulation evokes neural action potentials as seen in the intracellular recordings**.** Laser parameters: λ=3.65 μm, 30 Hz, 12.33 J/cm^2.

Discussion

This study presents the first results in the application of INS for CNS, and no previously published studies have continuously stimulated an *in Vitro* preparation using infrared light. Wilson et al. reported *in vitro* infrared stimulation of lobster nerves, but they were unable to maintain constant stimulation after one or two pulses of infrared light was applied to the tissue (A. R. Wilson et al., 2007). Additionally, there are no publications on infrared stimulation of the central nervous system, an area where high spatial precision is ideal. The results of this study show the ability to stimulate thalamocortical brain slices using infrared light reliably which was verified by blocking the signal by applying TTX to the perfusion chamber. The stimulation was accomplished without physical contact with the tissue and no stimulation artifact was evident on the electrical recording trace (figure 1); however the initial radiant exposures needed to evoke an extracellular potential resulted in ablation of the tissue. Ablative energies were needed because the top and bottom ~50 μm of each TC slice was dead due to the cutting process during slice preparation. Additionally, high energies were needed to recruit a sufficient number of neurons to evoke a measurable response. The TC slice contains

multiple neuronal networks where the infrared induced signal could travel. Since infrared stimulation is spatially precise, difficulty was experienced in detecting the signal electrically with a glass pipette electrode. These factors were taken into consideration while optimizing the parameters for INS stimulation of a TC slice, and each parameter was adjusted accordingly as more data was collected from INS stimulation of TC slices.

As was shown previously for the sciatic nerve, wavelength is a crucial parameter in INS (J. Wells et al., 2005a; J. Wells et al., 2005b; J. Wells et al., 2007a). The difference in threshold radiant energies at different wavelengths is attributed to the effective penetration depth (determined by the absorption coefficient at that wavelength) of the particular wavelength being investigated. Penetration depth is calculated based on the absorption coefficient for tissue of the wavelength of light being used for INS. Hence the wavelengths of light with lower absorption coefficients and higher penetration depths stimulated with lower radiant thresholds when compared with wavelengths of light with high absorption coefficients and low penetration depths. The optimal wavelength of light was identified as 3.65 μm which had an effective penetration depth of 344 μm. The penetration depth of 3.65 μm allows for optimal deposition of energy within the layer of living tissue minimizing the amount of energy deposited in the dead layers of tissue. The other wavelengths tested either had penetration depths that exceeded the depth of the tissue or had a shallow penetration depth which prevented the majority of energy to be absorbed in the superficial 50 μm layer of dead tissue. Energy delivery of infrared light to the living 300 μm layer of tissue was crucial for obtaining electrical recordings of stimulation events while performing INS. A point should be made that for future *in Vivo* studies, the 50 μm layer of dead tissue will not be present.

The central nervous system processes information at a higher rate when compared with the peripheral nervous system requiring higher repetition rates when performing electrical stimulation (C. D. Salzman et al., 1990; I. Stepniewska et al., 2005; E. J. Tehovnik et al., 2006), and Izzo et al. showed the need for higher repetition rates when using INS on the cochlea and the auditory vestibular nerve to transfer stimulated sensory information to the brain (A. D. Izzo et al., 2007; A. D. Izzo et al., 2008). Therefore, INS should function at a higher repetition rate in the central nervous system than what was found in the sciatic nerve during the original development of INS (J. Wells et al., 2007b; J. Wells et al., 2007c). The results showed that as repetition rate of the FEL increased, the threshold radiant energy decreased (Figure 3). At each repetition rate tested, a one to one response was recorded when related to the pulses of the laser. The higher frequency is most likely due to the need to overcome the intrinsic activity to produce a recognizable signal on the recording electrode to realize the one to one relationship and also deliver an appropriate average power to establish the appropriate heat gradient. The FEL could only generate light at a frequency of 30 Hz , which could indicate even lower radiant exposures needed to stimulate TC slices with higher frequencies; however no laser system was available at the time which could provide enough energy to induce stimulation at higher repetition rates.

The number of neurons recruited by INS affects the magnitude of the evoked signal measured using extracellular recording techniques. The last major parameter for INS in the TC slice was determining the optimal spot size of the laser beam. The results show that a larger spot size yields a lower threshold radiant exposure needed to stimulate where the data exhibited a power fit (Figure 4). The number of stimulated neurons is

dependent on spot size which can be explained by the high spatial precision of INS and the lack of current spread seen with electrical stimulation. The smaller spot size exposes a lower number of neurons to the light and also concentrates the energy from the laser into a small location normally causing significant ablation before reaching stimulation threshold; whereas, the larger spot size exposes a larger number of neurons to the light and distributes the light over a larger area allowing stimulation to occur at low radiant energies. In the TC slice, a small spot size was a limiting factor for evoking action potentials in the neuronal network. Further studies are needed in an *in Vivo* preparation where only living tissue will be present at the surface of brain, however detecting the induced signal will be limited due to the point source recording electrodes with a limited detection range of surrounding neurons. A more versatile detection system is needed to fully understand the limitations of the spatial precision associated with INS. Two such options are optical imaging techniques and functional magnetic resonance imaging. Both methods allow for a wider field of view and detection range when compared to traditional recording techniques, and both methods are non invasive since no electrodes are needed to impale the brain matter to detect signal.

INS intracellular recordings allowed for the viewing of the induced signal as it traveled through the neuronal network. Showing the ability to perform intracellular recordings while using INS is important for future scientific studies to further investigate how neurons process information and relay instructions to different regions of the body. A major advantage of INS over electrical stimulation in intracellular recordings is the lack of stimulation artifact seen in the recording electrode. The stimulation artifact seen with electrical stimulation will mask the biological signal hindering the investigator from

fully understanding the biology of the neurological signals being studied. With INS the stimulation artifact is removed revealing the signal that was previously masked to the investigator allowing for better characterization and understanding of the biology associated with signal transduction in the CNS. Additional experiments performed to obtain intracellular recordings proved difficult with the current set up due to logistical constraints of working with the FEL; however a small number of intracellular recordings were achieved at 3.65 μm and 4.00 μm (Figure 5). These recordings demonstrate the feasibility of performing INS with electrical intracellular techniques.

A common issue with using TC slices as a neuronal network model was the presence of a superficial 50 μm layer of dead tissue that are caused by the slicing method to prepare the slices. This layer must be penetrated to efficiently stimulate with INS and perform intracellular recordings. Since absorption is dependent on Beer's law and exhibits exponential decay, a large amount of energy was absorbed in the dead tissue layer before it could reach the living tissue increasing the threshold radiant energy needed to stimulate with INS. A set of experiments designed to remove the top 50 μm of tissue from the slice allowed for the radiant threshold energy for extracellular recordings to be dropped to 0.613 J/cm^2. At this energy, no tissue damage occurred indicating that removal of the superficial layer of dead tissue from the TC slice allows for safe efficient stimulation using infrared light; however logistical issues with the FEL lab prevented fine control of the laser beam to efficiently control the beam properties to further optimize a technique for removing the dead tissue layer from TC slices. Development of a bench top laser system is needed to optimize the removal of the dead tissue layer from the TC slice.

The results of this study show that infrared neural stimulation is possible in the central nervous system, specifically the brain. Results show consistent frequency locked stimulation and the ability to continuously stimulate over a long period of time. Infrared neural stimulation in the central nervous systems maintains the contact free, artifact free, and spatial precision shown in INS of the PNS (I. U. Teudt et al., 2007; J. Wells et al., 2007a; A. R. Wilson et al., 2007; A. D. Izzo et al., 2008; M. F. Nathaniel et al., 2008). These results present a strategy for future studies will focus on advancing the technique to an *in vivo* preparation using optical imaging as a detection technique. With the completion of the *in vivo* studies, more specific application based studies can advance INS to deep brain stimulation applications, more spatially precise cortical mapping, and application in neural-machine interfaces for neural prosthetics.

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CHAPTER III

FUTURE DIRECTIONS

Infrared neural stimulation (INS) has been characterized for the central nervous system *in Vitro*. Electrical responses were measured in the thalamocortical brain slice model using traditional electrophysiological techniques. Results from this study indicated that the optimal wavelength for stimulating the central nervous system was 3.65 μm where average stimulation threshold was determined to be 1.86 J/cm^2 for a spot size of 300-400 μm with ablation threshold determined to be approximately 1.1 J/cm^2. Stimulation threshold was shown to decrease to 0.66 J/cm^2 when the superficial dead layer of tissue in the brain slice was removed allowing for safe stimulation with no ablation of tissue. Additionally, a frequency and spot size dependence was established using the optimal wavelength of light at 3.65 μm. A small number of intracellular recordings were obtained which showed feasibility for using INS in further *in Vitro* models, specifically cultured cortical neurons. Results from this study have led to new studies to further investigate the uses of INS in the central nervous system. Specifically, a research plan will be developed to optimize the parameters of INS for *in Vivo* neural stimulation for my PhD dissertation.

The overall goal of the INS project is to improve upon current electrical techniques used to diagnose and treat neurological disorders. Development of INS in the central nervous system is crucial due to the vast possible applications and advantages of INS in treating and diagnosing disorders of the central nervous system. We therefore

propose to conduct a detailed parametric study to identify the optimal parameters of INS for stimulating the cortex of the brain. Due to difficulties of recording light induced action potentials using traditional electrical techniques, we will assess using intrinsic optical imaging techniques to detect neuronal activation evoked by INS in an initial feasibility study. Optical imaging will allow for high spatial resolution (~10 μm) while viewing a large area of interest which will eliminate the difficulty of placing electrical electrodes in the right place for detecting the optically induced neural signals. The parametric study will then be conducted to identify the optimal wavelength for *in Vivo* stimulation followed by studies to identify the optimal pulse width, repetition rate, and spot size for acute stimulation. Feasibility studies will then be conducted using the optimal parameters determined for acute stimulation to investigate the efficacy of chronic stimulation. The results of these studies will then be used to develop a table top laser system designed to stimulate different tissue structures of the brain both acutely and chronically. Once a system designed to stimulate the brain is in hand, clinical investigations will be used to assess the efficacy of INS for treating or diagnosing a number of neurological disorders.

All current techniques used to diagnose and treat neurological disorders use electrical stimulation techniques. Electrical stimulation requires touching of the neural tissue which can damage the tissue. Additionally, current spread from electrical stimulation limits the spatial precision of the technique. INS directly addresses these limitations of electrical stimulation by providing both contact free spatially precise stimulation method. These advantages of INS can improve upon traditional electrical

techniques in a number of specific areas including, deep brain stimulation, cortical mapping, and implementation in neural prosthetics.

Deep brain stimulation is used to treat tremor disorders (Parkinson's and Essential tremor), seizure disorders (epilepsy), and pain. Electrical deep brain stimulation often results in side effects while correcting or alleviating the treated symptom. These side effects can result in the patient perceiving pain, slight paralysis, or altered perception of senses to name a few; however the side effects caused by deep brain stimulation treatment are acceptable since the technique alleviates the more serious symptom which is debilitating. The side effects of deep brain stimulation are often due to current spread outside of the area where stimulation alleviates the symptom. Hence, INS can eliminate the risk of current spreading, which will prevent the formation of side effects allowing the patient to have a better quality of life while receiving INS deep brain stimulation treatment.

Cortical mapping is often used by a neurosurgeon to identify highly critical areas (speech, hearing, perception areas, etc.) during tissue resections to remove diseased brain tissue (epileptic tissue or tumors). High spatial precision is important when performing cortical mapping where microns of tissue removal can result in the problem still being present or result in debilitating side effects from the surgery. Currently cortical mapping is limited by the poor spatial precision of electrical stimulation due to current spread activating regions outside the stimulation zone. INS allows for stimulation of cortical areas of approximately 400 μm and does not requiring touching or impaling the cortex to evoke a response. Once fully developed, INS is expected to allow neurosurgeons to discriminate critical cortical areas surrounding diseased tissue with better precision

improving success of resection surgeries in removing tissue while preserving the maximum quality of life for the patient and preventing relapse of disease.

One area which stands to directly benefit from INS is the development of neural prosthetics for restoring function to limbs with damaged neural innervation as well as the possibility of coupling prosthetic devices to sensory regions in the brain to reestablish perception of specific sensations. People are commonly disabled due to damage suffered to neural innervation to a limb or a specific muscle group removing input from the brain resulting in paralysis. INS could potentially be used in an implantable device to where traditional electrical recording techniques are used to sense input from the brain which would then be transduced into pulses of infrared light from a chip containing an array of diode laser. Each diode would be coupled to individual fascicles which innervate individual motor units making it possible to bridge lesions in the nerve. An additional application of INS in neuroprosethics would deliver signals to the brain using light. Sensors could be implanted into the body to "sense" lost sensations such as taste, smell, sight, hearing, and balance. The signals could then be transduced into pulses of light to deliver the information directly to the areas of the brain used to integrate and interpret the specific sensation in a normal situation. The high spatial precision of INS will allow for a higher concentration of "optrodes" to be implanted when compared to electrodes in electrical stimulation resulting in better discrimination of the sensation components. Implementation of these applications of INS would require significant amount of research to miniaturize the technology and implement the necessary algorithms to perform the required tasks to restore function or perception to normal levels; however the potential for such applications exists and could be possible in the near future.

INS has remarkable potential for impacting the techniques used in neurosurgery to treat and diagnose patients with neurological disease and deficits. Currently INS of the CNS has been shown and optimized for *in Vitro* stimulation of the thalamocortical brain slice, but feasibility *in Vivo* experiments have shown promising results. The immediate future direction is the optimization of parameters for *in Vivo* INS followed by efficacy studies for chronic applications. Successful completion of these studies will lead to clinical studies which will use INS to improve on current electrical techniques used in neurosurgery. Immediate applications will improve upon DBS and cortical mapping techniques which are expected to benefit directly from the high spatial precision of INS. Advanced applications will involve the development instrumentation which will use INS to reestablish motor function to paralyzed limbs and perception for lost senses. The possible applications of INS are limitless which motivate the importance for the full development of INS as an activation method of neurons.